

## T-cell receptor-bearing cells from athymic nude rats respond to alloantigen *in vitro* but are defective *in vivo*

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### SUMMARY

T-like cells from congenitally athymic nude rats of the PVG (RT1<sup>c</sup>) strain were characterized both phenotypically and functionally. There was an age-dependent increase in the number of  $\alpha\beta$ TcR<sup>+</sup>CD3<sup>+</sup> cells in the lymph nodes, spleen and thoracic duct of nude rats. These cells, which comprised up to 25% of lymph node cells in animals of 8–12 months in age, were also CD3<sup>+</sup>CD5<sup>+</sup>Thy-1.1<sup>−</sup>. The expression of CD4 and CD8 on T-like cells was mutually exclusive. Approximately 30% of the CD4<sup>+</sup> cells expressed CD45RB and 50% of the TcR<sup>+</sup> cells expressed RT6. B-cell-depleted TcR<sup>+</sup> cells from nude animals gave proliferative responses to mitogenic lectins or immobilized anti-CD3 antibody. T-like cells showed comparable alloreactivity to their euthymic counterparts in mixed lymphocyte reactions (MLR) against three different MHC haplotypes and to lymphocytes of a congenic strain differing only in MHC-encoded products. Monoclonal antibodies (mAb) to CD4, MHC class II,  $\alpha\beta$ TcR and CD3 blocked the MLR. However, T-like cells failed to induce rejection of skin allografts of the same MHC haplotypes when adoptively transferred to athymic nude hosts and were unable to mount a normal graft-versus-host (GVH) response. These results indicate that lymphocytes may rearrange and express a functional TcR in the absence of a thymus, but that the thymic microenvironment is essential for T cells to acquire full *in vivo* function.

### INTRODUCTION

Congenitally athymic nude mice and rats have been widely used as animal models of T-cell deficiency.<sup>1–7</sup> Although a thymic rudiment is present, it never acquires or produces lymphoid cells.<sup>1</sup> However, an age-dependent increase in the number of cells bearing the phenotype of T cells has been consistently reported in these animals.<sup>8–10</sup> Furthermore, rearrangements of the genes coding for  $\alpha\beta$  TcR have been demonstrated in nude mice.<sup>11,12</sup> These and other observations led to the view that nude animals can develop T cells extrathymically, but very inefficiently<sup>13</sup> and that they, therefore, suffer essentially from a quantitative deficit of T cells. *In vitro* studies on the functional capacity of T-like cells have yielded contradictory results. Whilst some authors have reported an age-dependent increase in mitogen responsiveness of T-like cells,<sup>10,13,14</sup> others have failed to show any response, even when using purified cell populations.<sup>15,16</sup> A decisive *in vivo* demonstration of T-cell function in nude animals has been elusive.<sup>10,13</sup> It is unclear whether this lack of responsiveness *in vivo* is due to a qualitative or a quantitative deficiency.

In the present study, T-like cells bearing the  $\alpha\beta$ TcR were purified from lymph node cells (LNC) of aged nude rats. The

purified cells were compared on a cell to cell basis with equivalent cells from euthymic donors using both *in vitro* (lectin stimulation and MLR) and *in vivo* criteria (graft-versus-host activity and skin allograft rejection). We sought to establish whether the TcR<sup>+</sup> cells developing in the complete absence of a thymus were the equivalent of a thymus-derived product. This, in turn, would indicate whether full differentiation of T cells is ultimately dependent on the thymic microenvironment.

### MATERIALS AND METHODS

#### *Rats*

The following inbred and congenic strains of rat were bred and maintained in the Animal Unit, University of Manchester Medical School: AO (RT1<sup>u</sup>), BN (RT1<sup>b</sup>), DA (RT1<sup>a</sup>), PVG (RT1<sup>c</sup>), PVG rnu/rnu (nude, RT1<sup>c</sup>) and PVG-RT1<sup>u</sup>. PVG-R1 (RT1A<sup>a</sup>) rats were purchased from Olac Ltd, Bicester, Oxon, U.K. Nude rats were maintained under conventional conditions, but on a 19-week cycle of prophylactic antibiotic treatment as reported previously.<sup>17</sup> The colony of nude rats originated from a single pair of heterozygous animals back-crossed three times to PVG. Animals of 8–12 months in age were used in the present series of experiments unless otherwise specified. Euthymic heterozygous animals (PVG-rnu/+ ) were used as controls; when available, euthymic littermates were selected in preference.

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### Antibodies

The following monoclonal antibodies (mAb) were used for phenotyping, cell separation and *in vitro* studies: R73 (anti- $\alpha\beta$ TcR),<sup>18</sup> 1F4 (anti-CD3),<sup>19</sup> W3/25 (anti-CD4), OX19 (anti-CD5), W6/32 (anti-human HLA, control), OX8 (anti-CD8), OX12 (anti-rat  $\kappa$  chain), OX6 (anti-MHC class II), OX34 (anti-CD2), OX22 (anti-CD45RB), OX21 (anti-human C3b inactivator, control), P4/16 (rat anti-RT6), OX7 (anti-Thy-1.1). All mAb were obtained from Serotec, Bicester, Oxon, U.K., with the exception of 1F4 which was a kind gift from Dr T. Tanaka, Tokyo Metropolitan Institute, Tokyo, Japan. Ascites were used at 1/100 dilution and supernatants were used undiluted. For *in vitro* studies azide was removed from ascites by passing through a small G25 Sephadex column (Pharmacia, Uppsala, Sweden). FITC anti-rat immunoglobulin (Wellcome, Beckenham, Kent, U.K.) was used to detect surface immunoglobulin on rat B cells. mAb were purified using a MabTrap Protein G Sepharose column (Pharmacia) and biotinylated (bio-) according to Hardy<sup>20</sup> or conjugated to FITC as described by Goding.<sup>21</sup>

### Fluorescence staining and FACS analysis

Cells were stained as previously described.<sup>22</sup> Dual fluorescence staining was either a 2-stage procedure: (i) FITC-mouse mAb + bio-mAb, (ii) phycoerythrin-streptavidin (PE-SA; Becton-Dickinson, Mountain View, CA); or a 5-stage procedure: (i) mouse mAb, (ii) FITC F(ab')<sub>2</sub> anti-mouse Ig (Dako, High Wycombe, Bucks, U.K.) + 1% normal rat serum, (iii) W6/32 (blocking step), (iv) bio-mouse mAb, (v) PE-SA. Stained cells were fixed in 1% formaldehyde and analysed using a Becton-Dickinson FACScan.

### Isolation of TcR<sup>+</sup> cells

TDL (thoracic duct lymphocytes) were obtained by cannulation of the thoracic duct as previously described.<sup>17</sup> Cervical and mesenteric lymph nodes were excised and passed through a stainless steel mesh to obtain cell suspensions (LNC). B cells were stained with OX6 and OX12 and removed by mixing with anti-mouse Ig-coated ferric bio-mag particles (Advanced Magnetism Inc., Piddington, Northampton, U.K.). Particles with bound cells were separated using a samarium-cobalt magnet (Magnetic Developments Ltd, Swindon, Wilts, U.K.) as detailed previously.<sup>23</sup>

### Mitogen stimulation

B-cell depleted LNC were cultured at  $1.25 \times 10^6$  cells/ml in flat-bottomed 96-well clusters in a total volume of 200  $\mu$ l complete medium (RPMI-1640, Gibco, Grand Island, NY) containing 10% foetal calf serum (Seralab, Crawley Down, W. Sussex, U.K.), 1 mM glutamine, 10 mM HEPES,  $5 \times 10^{-6}$  M 2-mercaptoethanol, and 50 IU each of penicillin and streptomycin (Gibco). Mitogens were added at the following concentrations: concanavalin A (Con A; Sigma), 6  $\mu$ g/ml; phytohaemagglutinin (PHA; Wellcome), 20  $\mu$ g/ml; pokeweed mitogen (PWM; Sigma), 10  $\mu$ g/ml. Wells were coated with 1F4 (anti-CD3, 1/1000 dilution of ascites) for 3 hr at room temperature, and washed three times in phosphate-buffered saline (PBS). To assess cell proliferation, cultures were pulsed with 1  $\mu$ Ci/well [<sup>3</sup>H]TdR (5 Ci/mM; Amersham International plc, Amersham, Bucks, U.K.) after 48 hr and harvested 20 hr later using a Multi-Mash 2000 harvester (Dynatech, Billingham, Sussex, U.K.). Radioactivity was determined by liquid scintillation counting.

**Table 1.** Phenotypic analysis of whole LNC (a) or B cell-depleted LNC (b) from aged nude or euthymic rats

Surface antigen	n	% positive $\pm$ SD	
		Nude	Euthymic
(a)			
$\alpha\beta$ TcR	8	21.7 $\pm$ 2.4	57.4 $\pm$ 8.3
CD3	4	27.6 $\pm$ 4.8	50.1 $\pm$ 1.7
CD4	4	19.5 $\pm$ 2.9	49.6 $\pm$ 5.2
CD5	4	21.0 $\pm$ 2.2	53.9 $\pm$ 4.7
CD2	4	23.8 $\pm$ 3.9	54.4 $\pm$ 2.8
CD8	4	6.6 $\pm$ 1.8	8.5 $\pm$ 3.0
CD4 <sup>+</sup> CD45R <sup>+</sup>	3	6.8 $\pm$ 1.0	39.6 $\pm$ 8.5
CD4 <sup>+</sup> CD45R <sup>-</sup>	3	13.5 $\pm$ 1.0	15.0 $\pm$ 6.6
sIg	3	75.9 $\pm$ 11.3	ND
Thy-1.1	3	8.8 $\pm$ 4.7	ND
RT6	3	8.9 $\pm$ 1.4	57.0 $\pm$ 12.0
(b)			
$\alpha\beta$ TcR	6	85.3 $\pm$ 1.5	97.0 $\pm$ 2.3
CD4	3	63.5 $\pm$ 5.3	70.2 $\pm$ 9.2
CD8	3	26.9 $\pm$ 8.4	26.8 $\pm$ 4.2
CD5	3	89.4 $\pm$ 1.8	ND
Thy-1.1	3	5.3 $\pm$ 2.3	6.6 $\pm$ 4.0
RT6	3	41.5 $\pm$ 2.6	85.5 $\pm$ 1.3
sIg	3	9.4 $\pm$ 2.5	1.3 $\pm$ 0.3

### Mixed lymphocyte reaction (MLR)

$2.5 \times 10^5$  B-cell depleted LNC were mixed with  $5 \times 10^5$  irradiated stimulator LNC in a final volume of 200  $\mu$ l complete medium. Cultures were pulsed with [<sup>3</sup>H]TdR after 72 hr and harvested 20 hr later.

### Skin grafting

Skin grafting was carried out as previously described.<sup>17</sup> Grafts were inspected daily to monitor rejection, which was taken as the time for 50% of the graft to become necrotic.

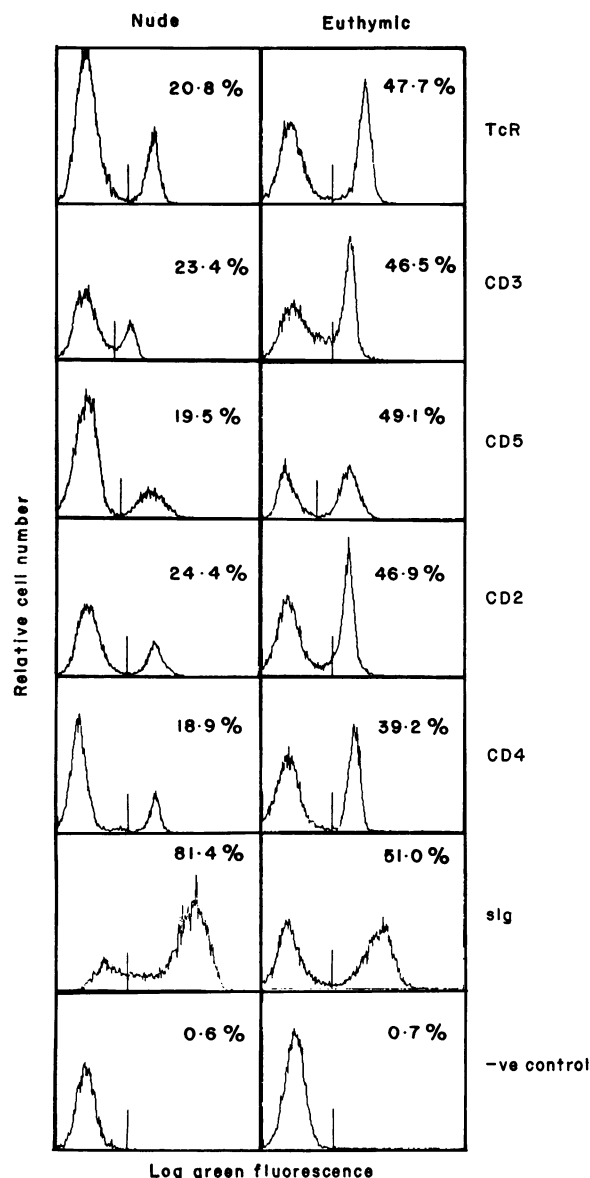
### GVH activity

GVH activity of LNC was measured by standard parental to F<sub>1</sub> popliteal lymph node (PLN) weight assay as described previously.<sup>17</sup>  $0.5-4 \times 10^6$  cells in 0.1 ml PBS were injected into the footpads of (PVG  $\times$  BN)F<sub>1</sub> or (PVG  $\times$  AO)F<sub>1</sub> recipients. Seven days later, PLN were removed and weighed. R1 values (the response in mg lymph node weight if a dose of 1 million cells had been injected) were calculated as previously described.<sup>17</sup>

## RESULTS

### Phenotype of LNC from aged nude rats

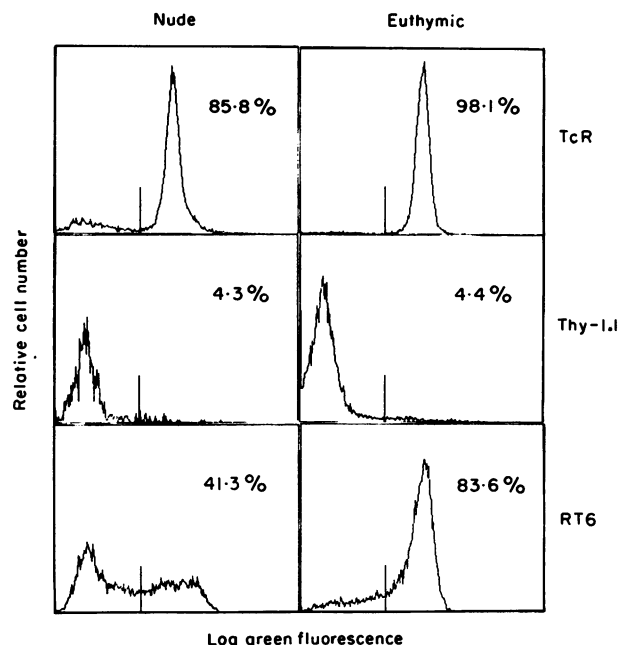
Phenotypic analysis of LNC from aged (8–12 months) athymic nude rats showed that approximately 22% of LNC were  $\alpha\beta$ TcR<sup>+</sup> cells (Table 1, Fig. 1). A similar proportion were CD3<sup>+</sup> CD5<sup>+</sup> CD2<sup>+</sup> CD4<sup>+</sup> (Table 1, Fig. 1). Although it is possible that a small population of CD3<sup>+</sup> CD5<sup>+</sup>  $\alpha\beta$  TcR<sup>-</sup> cells, which was recently proposed to be  $\gamma\delta$  TcR<sup>+</sup>,<sup>24</sup> was present, this population constituted <3% of total LNC and could not clearly be distinguished from background. One-third of the CD4<sup>+</sup> cells were CD45RB<sup>+</sup>, whereas in euthymic rnu/+ littermates 2/3 of



**Figure 1.** Phenotypic analysis (FACS profiles) of LNC from aged nude or euthymic rats. Percentages refer to positively staining cells to the right of the marker.

the CD4<sup>+</sup> cells were CD45RB<sup>+</sup> (Table 1, Fig. 3), indicating that the ratio of CD45RB<sup>+</sup>/CD45RB<sup>-</sup> subsets was reversed in nude animals.

The low levels of CD8, Thy-1.1 (which is expressed only on thymocytes and recent thymic emigrants in the rat) and RT6 (peripheral T-cell alloantigen Pta A2) made it difficult to assess the percentage of cells bearing these markers. Therefore the analysis was repeated on B-cell-depleted LNC (Table 1, Fig. 2). A significant percentage of sIg<sup>-</sup> cells expressed CD8 and RT6, although very few, if any, expressed Thy-1.1. The proportions of CD4<sup>+</sup> and CD8<sup>+</sup> cells in nude animals were similar to those in euthymic animals. Dual staining analysis showed no firm evidence of double positive CD4<sup>+</sup>CD8<sup>+</sup> or double negative CD4<sup>-</sup>CD8<sup>-</sup> TcR<sup>+</sup> cells (Fig. 3). The proportion of sIg<sup>-</sup> cells expressing RT6 in nude animals was about half that in euthymic animals (Table 1, Fig. 2).



**Figure 2.** Phenotypic analysis (FACS profiles) of sIg<sup>-</sup> LNC from aged nude or euthymic rats. Percentages refer to positively staining cells to the right of the marker.

Although depletion of B cells resulted in relatively pure populations of TcR<sup>+</sup> cells (>97% R73<sup>+</sup>) from euthymic LNC, when identical procedures were carried out on nude LNC, yields of approximately 85% R73<sup>+</sup> cells were consistently obtained. This difference could partially be accounted for by the higher proportion of contaminating sIg<sup>+</sup> B cells in the nude-derived population, however we cannot exclude that a subpopulation of TcR<sup>-</sup>sIg<sup>-</sup> cells was also present. Backgrounds of 3–6% were obtained with FITC anti-mouse Ig alone on B-cell depleted nude LNC compared to 1–2% for euthymic derived cells. mAb R73 also stained 10–17% of splenocytes and 20–25% of TDL in aged (8–12 months) nude rats. Only 4–6% of LNC or TDL from young nude rats (2–4 months old) were R73<sup>+</sup>.

### Mitogen responsiveness

B-cell depleted nude and euthymic LNC exhibited similar levels of responsiveness to the lectins Con A and PHA (T-cell mitogens) and PWM (mitogenic for both B and T cells). The response to cross-linked anti-CD3 mAb was also similar for cells from nude and euthymic animals (Fig. 4).

### MLR

Nude-derived TcR<sup>+</sup> cells showed a significant proliferative response to BN, AO, DA and the congenic strain PVG-RT1<sup>u</sup> (which differs from the background strain at the MHC locus only) (Fig. 5). In contrast, only a low response to self was observed. In all rats tested ( $n=5$ ), there was a consistent hierarchy of responsiveness: BN > AO > DA. The response to the congenic strain PVG-RT1<sup>u</sup> was similar to that to AO (RT1<sup>u</sup>), indicating that the response was generated by differences at the MHC locus. Euthymic cells gave MLR results which were entirely comparable qualitatively and quantitatively with TcR<sup>+</sup>

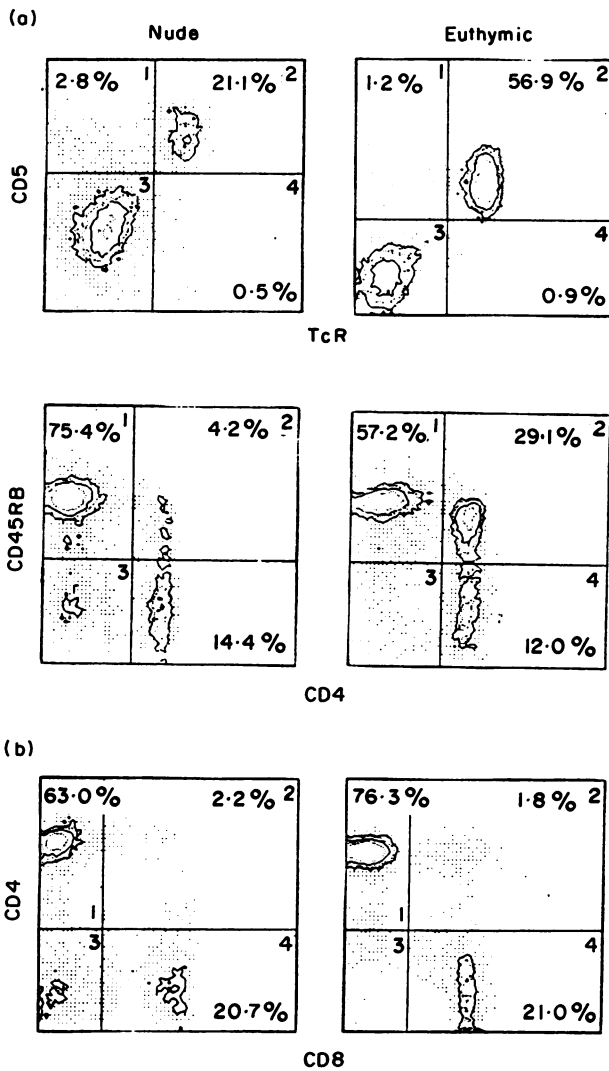


Figure 3. Dual-colour cytofluorographic analysis of (a) unseparated LNC or (b) slg<sup>-</sup> LNC of aged nude or euthymic rats. x axis=log green fluorescence (FITC); y axis=log red fluorescence (PE). Lowest level shown in contour plots=5 cells with graduations of 5, 15 and 25 cells.

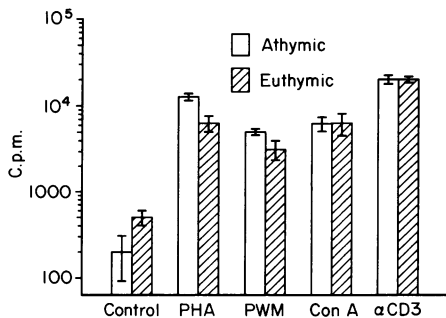


Figure 4. Mitogen responsiveness of TcR<sup>+</sup> cells from aged nude rats. [<sup>3</sup>H]TdT incorporation is expressed as c.p.m. for each added mitogen. Medium alone was added to control cultures. Results are means of five experiments  $\pm$  SD.

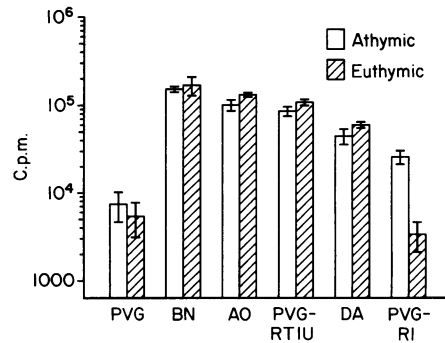


Figure 5. MLR of TcR<sup>+</sup> cells from aged nude rats or euthymic (PVG rnu/+ ) rats. [<sup>3</sup>H]TdT incorporation is expressed as c.p.m. for PVG rnu/rnu or PVG rnu/+ responders with irradiated stimulator LNC from rats of the strains indicated. Results are means of five experiments  $\pm$  SD.

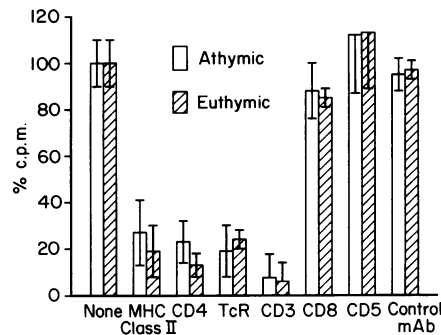


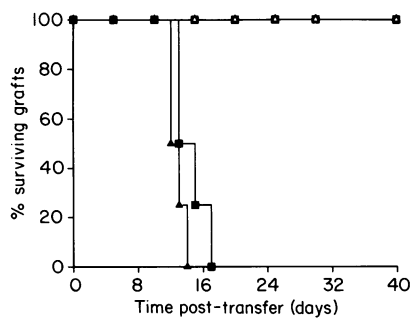
Figure 6. Effect of blocking mAb on the MLR of TcR<sup>+</sup> cells from aged nude (PVG-rnu/rnu) or euthymic (PVG-rnu/+ ) rats. Stimulator cells were irradiated LNC of the BN strain. [<sup>3</sup>H]TdT incorporation (c.p.m.) is expressed as percentage of the control MLR with no mAb added (100%). Specificities of added mAb are shown on the x axis. Results are means of four experiments  $\pm$  SD.

nude derived cells. Reactivity against the congenic strain PVG-R1 (an MHC class I only disparity) was low for the nude derived TcR<sup>+</sup> cells. This response was, however, significantly greater than that of the euthymic cells, which had levels of reactivity similar to the autologous control. Cultures with both irradiated responders and stimulators showed background levels of proliferation.

The MLR was significantly decreased for both athymic and euthymic TcR<sup>+</sup> cells by the addition of the mAb against MHC class II, CD4,  $\alpha$ TcR and CD3, but not by mAb against CD8, CD5 or by negative control mAb OX21 (anti-human C3b inactivator) (Fig. 6).

#### Skin allograft rejection

Previous work has shown that skin allografts were rejected by nude recipients only following the adoptive transfer of CD4<sup>+</sup> T cells.<sup>23</sup> The analysis of alloreactive CD4<sup>+</sup> cells in these nude recipients indicated that they were of donor (euthymic) origin.<sup>25</sup> The speed of rejection was dose-dependent up to  $5 \times 10^6$  CD4 T cells; as few as  $10^5$  CD4 cells were sufficient. In order to assess the ability of athymic CD4<sup>+</sup>TcR<sup>+</sup> cells to induce rejection directly,



**Figure 7.** TcR<sup>+</sup> cells from nude rats fail to induce skin allograft rejection. PVG nude recipients (five in each group) were double grafted with BN (▲, △) and AO (●, ○) skin grafts and injected 8 days later with B-cell-depleted sIg<sup>-</sup> LNC from nude (open symbols) or euthymic (closed symbols) donors.

**Table 2.** GVH responsiveness of TcR<sup>+</sup> cells obtained from aged nude and euthymic rats

F <sub>1</sub> hybrid recipient	GVH (R <sub>1</sub> values)* calculated for:			
	TcR <sup>+</sup> cells		CD45R <sup>+</sup> cells	
	Nude	Euthymic	Nude	Euthymic
PVG × BN	8.6	34.4	16.2	48.9
PVG × AO	10.5	23.5	12.3	29.3

\* Values are mean PLN weights in mg for an injected dose of  $1 \times 10^6$  cells.

Mean PLN weights for injection of PBS alone or  $1 \times 10^6$  syngeneic (F<sub>1</sub>) cells were 4.9 and 3.7 mg, respectively.

$4 \times 10^6$  B-cell-depleted TDL from aged euthymic or nude donors were transferred into young nude recipients bearing established BN and AO skin grafts. This approach allowed a direct comparison of the efficacy of equal numbers of nude and euthymic-derived cells in skin graft rejection.

BN and AO skin allografts were promptly rejected by nude rats injected with  $4 \times 10^6$  sIg<sup>-</sup> TDL from euthymic donors (Fig. 7). The rejection times were: BN, 12–14 days; AO, 13–17 days. In contrast, animals injected with the same number of sIg<sup>-</sup> TDL from nude donors failed to reject either BN or AO skin allografts (Fig. 7). All of the latter animals died within 40–55 days of transfer of nude-derived TDL, with fully intact skin allografts still in place.

### GVH activity

GVH activities of LNC from aged nude and euthymic rats were tested in a PLN weight assay in both (PVG × AO)F<sub>1</sub> and (PVG × BN)F<sub>1</sub> hybrids (Table 2). In parallel with the hierarchy of MLR reactions, and also the speed of allograft rejection, euthymic LNC induced larger GVH responses against the BN than against the AO haplotype. TcR<sup>+</sup> nude-derived cells gave significantly lower ( $P < 0.001$ ) GVH responses against both haplotypes (Table 2). We and others<sup>26,27</sup> have shown that the

ability to induce GVH responses is greatly enriched in the CD4<sup>+</sup>CD45R<sup>+</sup> subset. Since the proportion of CD45R<sup>+</sup>:CD45R<sup>-</sup> CD4 cells is lower in the population obtained from nude animals (Table 1, Fig. 2), the GVH responses (i.e. R<sub>1</sub> values) were also calculated on the basis of CD45R<sup>+</sup> cells alone (Table 2). Again the results indicated that nude-derived TcR<sup>+</sup> cells were significantly deficient in eliciting GVH responses. The further question of whether nude-derived cells induced a positive GVH response at all is problematic. Weights of PLN draining the injection sites of nude cells (8.6 mg) were significantly larger ( $P < 0.02$ ) than that of PBS alone (mean = 4.9 mg) or that of syngeneic F<sub>1</sub> cells (3.7 mg). On the other hand, nude-derived cells did not evoke a dose-response weight increase that was in any way comparable with euthymic cells (results not shown); nor was there the usual differential response seen between BN and AO haplotypes. Thus, it was not clear whether or not the slightly enlarged PLN induced by nude-derived cells was the result of allorecognition or some other unidentified process.

### DISCUSSION

Although the normal pathway of T-cell development is through the thymus, it is important to establish whether T cells can also be generated, however inefficiently, elsewhere, or whether the thymus confers a seminal change on T-cell precursors, without which fully functional T-cell differentiation fails.

The present phenotypic investigation confirms and extends previous reports.<sup>8–13</sup> We observed lymphocytes of extrathymic origin, with surface molecules identical to those expressed in cells derived from the thymus; up to 25% of LNC in aged nude rats simultaneously expressed CD2, CD4, CD5, CD3 and the  $\alpha\beta$  TcR, the phenotype of mature T cells. There was no evidence of a significant population of cells with the phenotype of immature T cells: few, if any, cells stained brightly with the mAb OX7 (anti-Thy-1.1), which reacts selectively with thymocytes<sup>28</sup> and recent thymic emigrants in the rat (C. P. Yang and E. B. Bell, unpublished results). Moreover, no significant population of TcR<sup>+</sup> cells with the double-negative (CD4<sup>-</sup>CD8<sup>-</sup>) or double-positive (CD4<sup>+</sup>CD8<sup>+</sup>) phenotypes typical of intermediate stages in thymocyte development were observed. RT6, which is a marker of mature peripheral T cells<sup>29</sup> was also present, albeit on a lower percentage of cells than in euthymic animals. The lower percentage of RT6<sup>+</sup> cells resembled that observed in CD4 T-cell reconstituted nude rats,<sup>30</sup> in which normal T-cell responses were documented.

The normal 2:1 ratio of CD45R<sup>+</sup>:CD45R<sup>-</sup> CD4 T cells in euthymic animals was inverted in nude rats, confirming a recent report.<sup>31</sup> Until very recently<sup>26,30</sup> these restricted splice variants of the leucocyte common antigen (CD45) were thought to distinguish naive (CD45R<sup>+</sup>) from memory T cells (CD45R<sup>-</sup>).<sup>27</sup> Functionally the CD45R<sup>+</sup> subset was able to induce augmented MLR and GVH reactions,<sup>32</sup> whereas the reciprocal CD45R<sup>-</sup> population helped B cells produce antibody.<sup>32</sup> The adoptive transfer of purified subsets into nude rats showed that CD45R<sup>+</sup>/CD45R<sup>-</sup> CD4 T cells were interconvertible and could switch isoform expression and change function in both directions.<sup>26,30</sup> It is interesting that donor-derived CD4 T cells, which permanently restored the recirculating pool of nude recipients,<sup>17</sup> also

had an inverted CD45R<sup>+</sup>:CD45R<sup>-</sup> ratio, although the response of these animals to alloantigen was unaffected.<sup>30</sup>

When  $\alpha\beta$ TcR<sup>+</sup> cells were purified from nude rat LNC, they gave *in vitro* mitogenic responses and MLR (against three independent haplotypes) equivalent to those of euthymic T cells. Responsiveness was blocked by mAb to CD3, CD4,  $\alpha\beta$ TcR and MHC class II; alloantigens coded by the MHC alone induced responses. The *in vitro* studies were entirely consistent, therefore, with conventional allorecognition of MHC class II by CD4<sup>+</sup> T cells resulting in proliferation. The MLR cultures demonstrated that expression of a TcR capable of recognizing alloantigens occurred independently of the thymus. Whatever else the thymic microenvironment may do, it apparently does not control the productive rearrangement of the TcR. An unexpected difference between nude and euthymic cells was the small but significant MLR of the nude T-like cells to the congenic strain PVG-R1 (which differs from the PVG strain at class I only). The response of euthymic T cells was negligible. The reason for this differential responsiveness is unknown.

On the basis of restricted V $\beta$  TcR gene expression in nude mice<sup>12,33</sup> it was suggested that the specificity of the T-cell repertoire was oligoclonal, thus accounting for the functional defect in athymic animals.<sup>13</sup> The ability of the nude-derived TcR<sup>+</sup> cells to proliferate in MLR against three different MHC haplotypes does not support this argument. If indeed the TcR repertoire is oligoclonal, there nevertheless remains sufficient variability to permit normal allorecognition. The lack of an autologous response in this system also suggests that a mechanism of peripheral tolerance may be operative. The detection in the nude mouse of cells expressing potentially autoreactive TcR in the absence of overt autoimmune disease<sup>34,35</sup> suggested that self-reactive cells were inactive, rather than clonally deleted.

Despite their phenotype and *in vitro* responsiveness, TcR<sup>+</sup> cells from nude rats were unable to initiate skin allograft rejection and showed little, if any, capacity to induce a GVH response *in vivo* against the same antigens which evoked an MLR. This discordance between alloresponsiveness *in vivo* and *in vitro* was also noted following the implantation of allogeneic thymic epithelium into nude rats.<sup>36</sup> The T cells which developed in these animals (host origin) responded in MLR but were tolerant of skin grafts of the thymic epithelial haplotype while rejecting third party skin.

Since the inability of extrathymically derived TcR<sup>+</sup> cells to reject skin grafts could not be attributed to a failure to recognize relevant alloantigens, other explanations were considered: (i) an insufficient number of TcR<sup>+</sup> cells; (ii) lack of effector cells; (iii) a failure of cells to reach the allograft site; (iv) a fundamental defect in T cell differentiation.

(i) On the basis of our previously published data,<sup>23</sup> adoptive transfer of 4 million sIg<sup>-</sup> TDL of nude origin should be at least 40 times in excess of the number of T cells needed for skin allograft rejection, had the cells been the equivalent of those from the thymus. The argument that nude animals have inadequate numbers of T-like cells for rejection is not tenable. Even in young unreconstituted nude rats of, for example, 3–4 months in age, T-like cells comprise 4–5% of the recirculating lymphocyte pool, i.e. a total of  $2\text{--}2.5 \times 10^7$  T-like cells—over 200 times the number of euthymic T cells required to elicit skin allograft destruction. Yet these animals fail to reject skin allografts. Thus a quantitative difference cannot explain the inability of T-like cells to induce allograft rejection.

(ii) Adoptive transfer to nude recipients showed that highly purified CD4<sup>+</sup> TDL from euthymic donors were necessary and also sufficient to induce skin allograft rejection across a full MHC disparity.<sup>23</sup> Although the inducer cell was clearly a CD4<sup>+</sup> T cell, the identity of the effector cells causing allograft destruction is still uncertain. If the CD4 T-cell itself is not the effector cell, then a non-thymus-derived component must be responsible.<sup>23</sup> In the latter case, the nude animal must be adequately endowed with an effector mechanism. The success of adoptively transferred CD4<sup>+</sup> cells of euthymic origin in reconstituting T-cell responses in nude recipients also suggests that suppression or a failure in antigen presentation cannot account for the lack of T-cell function in unreplaced nude rats.

(iii) Could the inability of T-like cells to migrate to sites of alloantigen in either the afferent or efferent arms of the immune response explain their lack of *in vivo* reactivity? This seems unlikely since recirculating CD4<sup>+</sup> cells are found in the thoracic ducts of nude rats.<sup>17</sup> Moreover, TcR<sup>+</sup> cells of host (nude) origin have been demonstrated in the rejecting kidney allografts of nude rats reconstituted with T cells from euthymic rats,<sup>22</sup> although it has not been determined whether their entry into the graft was facilitated by vascular destruction occurring early in the rejection process.

(iv) Present evidence suggests that nude-derived TcR<sup>+</sup> cells are profoundly different from thymus-derived TcR<sup>+</sup> cells. Nude TcR<sup>+</sup> cells lack a vital differentiation step. The mere ability to proliferate following alloantigen recognition (e.g. in an MLR) apparently does not guarantee that this quintessential event has taken place, unless, of course, it actually occurs in culture during the generation of the MLR, which seems unlikely. Lack of thymus processing could result in cells which become tolerized *in vivo* by contact with alloantigen. Possibly the thymic microenvironment programmes T-cell precursors for particular lymphokine production or establishes an intracellular signal transduction pathway not otherwise open. Others have observed that TcR<sup>+</sup>CD3<sup>+</sup> thymocytes differed from mature T cells in their ability to mobilize intracellular Ca<sup>2+</sup>.<sup>37</sup>

IL-2 production by T-like cells, including an age-related increase corresponding with an increase in Thy-1<sup>+</sup> cells, was reported in nude mice.<sup>14,16</sup> In one study,<sup>16</sup> purified CD4 cells from nude mice were found to produce significant quantities of IL-2 and to express IL-2 receptors. The additional observation that nude-derived CD4 cells failed to divide, following stimulation with mitogenic lectins, anti-CD3 mAb or phorbol ester plus Ca<sup>2+</sup> ionophore, is at odds with the work of others<sup>10,14</sup> and the present study, for reasons which are unclear. Attempts to restore T-cell function by injecting athymic mice with preparations rich in IL-2 met with limited or no success.<sup>38,39</sup> On current evidence, IL-2 production does not appear to discriminate between CD4 cells that have developed intra or extrathymically.

Other lymphokines may be more important. The poor or absent GVH response of nude-derived cells is interesting in this regard. The GVH popliteal LN assay is generally considered to be a measure of allorecognition and correlates with the MLR. In the present investigation, however, these two assays gave discordant results. Aside from the obvious *in vitro* versus *in vivo* distinction, the two assays differ in one important aspect. Whereas the MLR measures direct donor (CD4) T-cell proliferation, the popliteal LN enlargement results primarily from a host B-cell expansion.<sup>40</sup> The latter assay thus detects the consequences of specific T-cell allorecognition, i.e. the T-cell

products released into the microenvironment, and not the proliferation of CD4 T cells directly. By implication, the nude-derived CD4 cells apparently failed to liberate the T-cell products required for B-cell proliferation *in vivo*. This observation may highlight an important characteristic that distinguishes thymus-derived from non-thymus-derived products.

The question of whether Tc cytotoxic cells may develop extrathymically in nude animals<sup>13</sup> was not addressed in the present study and is beyond the scope of this discussion. Elsewhere<sup>41</sup> we have stressed the need to re-evaluate the nature of the cytotoxic activity generated in nude animals so as to distinguish it from a second form of cytotoxicity effected by non-thymus-derived cells<sup>42</sup> that kill allogeneic target lymphocytes *in vivo* and *in vitro*—a system that also depends, like Tc cells, on recognition of MHC gene products.<sup>43</sup>

In conclusion, current evidence suggests that CD4<sup>+</sup>TcR<sup>+</sup> cells in nude rats are deficient and not the equivalent of CD4 T cells emigrating from the thymus. The thymic micro-environment appears to be an absolute requirement for the development of fully functional CD4 T cells. A search for the distinguishing features between intra and extrathymically derived CD4<sup>+</sup>TcR<sup>+</sup> cells should aid in understanding the critical differentiation events that occur in the thymus.

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